The transcriptional coactivator PGC-1α mediates exercise-induced angiogenesis in skeletal muscle

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Peripheral arterial disease (PAD) affects 5 million people in the US and is the primary cause of limb amputations. Exercise remains the single best intervention for PAD, in part thought to be mediated by increases in capillary density. How exercise triggers angiogenesis is not known. PPARγ coactivator (PGC)-1α is a potent transcriptional coactivator that regulates oxidative metabolism in a variety of tissues. We show here that PGC-1α mediates exercise-induced angiogenesis. Voluntary exercise induced robust angiogenesis in mouse skeletal muscle. Mice lacking PGC-1α in skeletal muscle failed to increase capillary density in response to exercise. Exercise strongly induced expression of PGC-1α from an alternate promoter. The induction of PGC-1α depended on β-adrenergic signaling. β-adrenergic stimulation also induced a broad program of angiogenic factors, including vascular endothelial growth factor (VEGF). This induction required PGC-1α. The orphan nuclear receptor ERRα mediated the induction of VEGF by PGC-1α, and mice lacking ERRα also failed to increase vascular density after exercise. These data demonstrate that β-adrenergic stimulation of a PGC-1α/ERRα/VEGF axis mediates exercise-induced angiogenesis in skeletal muscle.

VEGF | ERRα | β-adrenergic

The rising physical inactivity in Western societies is worsening the prevalence and severity of many chronic diseases, including obesity, diabetes, atherosclerosis, and neurodegenerative diseases. Exercise remains one of the most efficient interventions for most of these. Peripheral artery disease (PAD), in particular, is a leading cause of morbidity and the most common cause of limb amputation in the U.S., and yet even the best medical therapy available is less efficacious than simply walking daily (1, 2). Muscle adapts to endurance-type exercise by triggering mitochondrial biogenesis, changes in fiber composition, and the growth of new blood vessels, or angiogenesis (3–5). These changes in muscle composition carry out many of the health benefits of exercise. Angiogenesis, in particular, likely improves symptoms in PAD (6, 7). The efficient induction of angiogenesis in ischemic limbs has therefore long been a therapeutic goal (8, 9).

Angiogenesis, however, is a complex process (10, 11), and clinical trials have been hampered by the inability to induce the formation of completely functional vessels (11–14). One key shortcoming has been that the use of angiogenic factors like vascular endothelial growth factor (VEGF) appears to be insufficient for the generation of fully functional vessels. A number of other factors like PDGF's, angiopoietins, and various inhibitors, contribute to the complex remodeling events that occur during angiogenesis. Triggering and regulating angiogenesis is therefore not just a matter of secreting one or two factors, but instead requires a complete programmatic orchestration. Exercise is one of the few physiological processes that activates such an orchestrated angiogenic response in adults (7, 11, 15). Understanding the gene regulatory mechanisms that trigger angiogenesis in response to exercise is therefore of great interest.

Few data exist to address the molecular mechanisms underlying exercise-induced angiogenesis (16, 17). The prevailing notion has been that exercise-induced angiogenesis is triggered by the increased metabolic needs of active and newly oxidative muscle (7, 15–17). In this model, local hypoxia caused by prolonged exercise stabilizes the transcription factor hypoxia inducible factor-1α (HIF-1α), leading to the induction of VEGF and angiogenesis. However, hypoxia has been difficult to demonstrate in muscle undergoing endurance exercise (15), and deletion of HIF-1α in skeletal muscle increases, rather than decreases, microvascular density (18). The metabolic sensor AMP Kinase (AMPK) has also been hypothesized as another pathway, sensitive to metabolic insufficiency, which may mediate exercise-induced angiogenesis. However, mice transgenically expressing a dominant negative form of AMPK in skeletal muscle display normal increases in capillary density after exercise (19). How exercise induces VEGF and mediates exercise-induced angiogenesis therefore remains unclear.

The transcriptional coactivator PGC-1α is a dominant regulator of oxidative metabolism in many tissues, and has emerged as a protein of great interest in the science of bioenergetics (reviewed in refs. 20 and 21). Coactivators are proteins that dock on transcription factors and alter chromatin structure and the transcription machinery to stimulate gene expression (reviewed in refs. 22 and 23). Several coactivators are key regulatory targets of physiological stimuli and hormones, and PGC-1α is the best-studied example of such a regulated coactivator. PGC-1α powerfully regulates broad and comprehensive genetic programs in skeletal muscle, including the activation of fatty acid oxidation and oxidative phosphorylation, and the conversion of muscle fibers to an oxidative type (24, 25). Oxidative fibers are also rich in capillaries, and we recently showed that PGC-1α can induce angiogenesis in skeletal muscle, in a HIF-independent fashion (26). Here, we demonstrate that PGC-1α mediates exercise-induced angiogenesis, and investigate the mechanisms by which this occurs.

Results

Exercise-Induced Angiogenesis Requires PGC-1α. To investigate angiogenesis in skeletal muscle in response to exercise, we used an established model of voluntary endurance training. Eight-week-old mice were placed singly in cages equipped with electronically monitored running wheels. The mice were then allowed to use the wheels ad libitum. After an accustomization period of a few days, wild-type C57/Bl6 mice (which are nocturnal animals) ran the equivalent of 8 km or more per night, while resting during the day (Fig. S1A). At various times after initiation of voluntary running, the mice were killed and the quadriceps muscle was removed. Thin
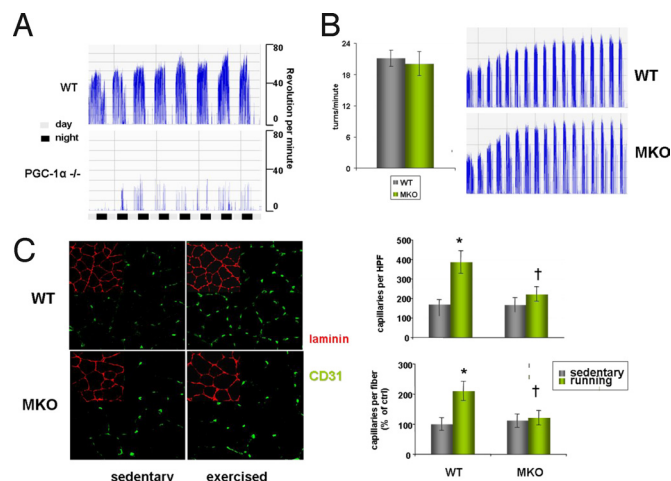
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Fig. 1. Exercise-induced angiogenesis requires PGC-1α. (*A*) PGC-1α −/− (total-body deletion) mice fail to run on in-cage running wheels. A sample tracing of wheel activity, in revolutions per minute, is shown for both WT and PGC-1α −/− mice. (*B*) Mice lacking PGC-1α specifically in skeletal muscle (MKO mice) do run on in-cage running wheels. *Right*, sample tracings of wheel activity. *Left*, average distance run. *n* = 5 per group. (*C*) Capillary density from wild-type and PGC-1α MKO mice, either after 14 days of voluntary running, or sedentary controls. *Left*, representative immunostains for CD31 (endothelial-specific PECAM) in green. *Insets* show immunostains for laminin of same section, highlighting muscle fiber outlines. *Right*, quantification of microvascular density. *n* = 5 per group. Data are presented as mean ± SEM. *, *P* < 0.05 vs. control. †, *P* < 0.05 vs. WT exercised.

Fig. 2. β-adrenergic signaling and exercise induce expression of PGC-1α from an alternative promoter. (*A*) Schema of PGC-1α alternative and proximal promoters. See text for details. Homology between mammalian species (rat, human, dog, horse, monkey, chicken) is indicated. (*B*) Tissue distribution of total PGC-1α mRNA (*Top*) and mRNA initiated at the alternative promoter (*Bottom*), as determined by qPCR. (*C*) Relative expression in quadriceps of total PGC-1α mRNA (*Left*), PGC-1α mRNA originating at the proximal promoter (*Middle*), or the alternative promoter (*Right*), after running on voluntary wheels for the indicated time. (*D*) Relative expression of total PGC-1α mRNA (*Left*), PGC-1α mRNA originating at the proximal promoter (*Middle*), or the alternative promoter (*Right*), 6 h after i.p. injection of clenbuterol (200 μg/kg). (*E*) Anti-PGC-1α Western blot analysis of quadriceps extracts from mice after 16 h of voluntary running (+) vs. control (−). NS, nonspecific band. (*F*) Anti-PGC-1α Western blot analysis of quadriceps extracts from WT and MKO mice 6 h after PBS (−) or clenbuterol (−) injection. NS, nonspecific band. (*G*) Expression of alternative PGC-1α in quadriceps, at the indicated time after clenbuterol injection. (*H*) PGC-1α expression 6 h after clenbuterol or saline injection in wild-type or PGC-1α MKO mice. Exons 3–5 are deleted in the MKO mice, while both the 3′ UTR and the alternative promoter remain intact. *n* = 3 per group for *A–H*. Data are presented as mean ± SEM. *, *P* < 0.05 vs. control. †, *P* < 0.05 vs. WT clenbuterol treated.

transverse sections were prepared, and capillaries were visualized by immunostaining with antibodies against CD31 (PECAM), a marker specific to the endothelial wall. As shown in Fig. S1*B*, capillary density in the midportion of the quadriceps was 2-fold greater in mice after 14 days of voluntary running versus matched sedentary controls. We restricted our examination to the midportion of the quadriceps because the most superficial portion has sparse capillary density that does not increase with exercise, likely because the superficial quadriceps is not heavily recruited during endurance running, while the deepest part of the quadriceps (abutting the femur) has a high capillary density even without exercise. These data demonstrate that voluntary exercise is a powerful angiogenic stimulus in rodent skeletal muscle, as has been reported (refs. 17 and 27).

To test the role of PGC-1α in exercise-induced angiogenesis, we initially sought to test the angiogenic response of PGC-1α −/− mice to voluntary running. However, as shown in Fig. 1*A*, PGC-1α −/− mice did not run appreciably on in-cage running wheels, despite being hyperactive and hypermetabolic at baseline (28). PGC-1α −/− mice display markedly abnormal behavior, and have a number of central nervous system lesions (28, 29), likely explaining their unwillingness to run on in-cage wheels. We therefore turned to mice lacking PGC-1α specifically in skeletal muscle. Mice in which exons 3–5 of the PGC-1α gene are flanked by Lox recombination sites (a kind gift of Bruce Spiegelman, Boston, MA) were bred with mice transgenically expressing the CRE recombinase under control of the skeletal muscle MEF2C enhancer and the myogenin promoter (30), to generate muscle-specific PGC-1α knockout (MKO) mice, as has been described elsewhere (31). PGC-1α MKO mice and littermate controls were then either provided with in-cage running wheels for 14 days, or placed in cages lacking running wheels, as control. As shown in Fig. 1*B*, MKO mice did willingly run when provided in-cage running wheels. Importantly, the MKO mice ran on average as much as did WT controls (Fig. 1*B*). It is interesting that MKO mice do perform somewhat more poorly than control mice when forced to run to maximum capacity on treadmills (31). In the current experimental protocol, however, mice run strictly

within their "comfort zone," since the wheels are voluntary. This likely reflects more closely the type of exercise regularly performed by humans. In this setting, there appears to be no difference in running between wild-type and MKO mice.

Capillary density in the quadriceps was then measured by immunostaining for CD31, as described above. In the absence of voluntary running, the capillary density in the quadriceps of PGC-1α MKO mice did not differ significantly from WT controls (Fig. 1*C*, gray bars), suggesting that either PGC-1α plays no role in baseline vascular density, or that redundant activities (e.g., PGC-1β) compensate for the absence of PGC-1α. After exercise, capillary density increased >2-fold in WT control animals (Fig. 1*C*, left bars). In sharp contrast, there was almost no increase in capillary density after exercise in MKO mice (right bars). Thus, PGC-1α is required for exercise-induced angiogenesis in skeletal muscle.

β-Adrenergic Signaling and Acute Exercise Induce Expression of PGC-1α from an Alternative Promoter.

Consistent with an important role for PGC-1α in exercise-induced angiogenesis, numerous studies have shown that PGC-1α expression in human and rodent skeletal muscle is strongly induced by exercise (e.g., refs. 32–34). The precise mechanism for this induction remains unclear. While investigating PGC-1α expressed sequence tags (ESTs) and cross-species homologies in public databases, we noted, as did others (35), the existence of a conserved, putative alternative promoter to PGC-1α, located approximately 14 kilobases upstream of the proximal promoter (Fig. 2*A*). Transcription from this alternative promoter, followed by one of two alternative splicing events, yields mRNAs with first exons that differ from transcripts started at the

proximal promoter. The alternative mRNAs predict the translation of two proteins, PGC-1α2 and PGC-1α3, that differ from the canonical PGC-1α (renamed PGC-1α1 here) in a few amino acids at their amino terminus (Fig. 2A).

To investigate whether transcripts initiated at this alternative promoter are expressed, and in what tissues, cDNAs were prepared from a variety of tissues, and expression from the two promoters was quantified using quantitative real-time PCR (qPCR) and primers specific to transcripts from either promoter. As previously reported, total PGC-1α mRNA expression was greatest in highly metabolic tissues, such as kidney, heart, and BAT (Fig. 2B Top). In contrast, while mRNAs initiated at the alternative promoter were relatively abundant in muscle (heart and skeletal) and brown adipose tissue (BAT), other tissues had nearly undetectable amounts of transcripts initiated at the alternative promoter (2B Bottom). Relative expression from the alternative promoter in different muscle beds paralleled expression from the proximal promoter (Fig. S2). Hence the alternative promoter appears to be uniquely active in muscle and BAT.

Next, we investigated the effects of exercise on mRNA expression from either promoter. Wild type mice were allowed to run on in-cage running wheels for 0, 4, or 16 h, and mRNA expression was measured by qPCR. As shown in Fig. 2C, total PGC-1α expression, as determined using primers common to transcripts initiated from both PGC-1α promoters, was induced 8-fold by exercise. Strikingly, however, expression of PGC-1α mRNA from the proximal promoter was not induced at all, while expression from the alternative promoter increased >100-fold. Western blot analysis of muscle extracts from sedentary and exercised mice demonstrated strong induction of PGC-1α (Fig. 2E). Hence, the increase in PGC-1α expression seen after exercise stems entirely from the alternative promoter.

Miura et al. (36) recently showed that stimulation of β-adrenergic receptors in skeletal muscle leads to a dramatic induction of PGC-1α expression. Furthermore, the induction of PGC-1α seen after an acute bout of exercise was at least in part dependent on β-adrenergic signaling. To investigate the effects of β-adrenergic signaling on the alternative PGC-1α promoter, wild-type mice were injected intraperitoneally with clenbuterol (200 μg/kg), a long-acting β2 agonist, versus saline control. Six hours later, RNA was prepared from quadriceps muscles, and expression of PGC-1α was measured by qPCR. As shown in Fig. 2D, total PGC-1α expression was induced >5-fold by clenbuterol. Induction from the alternative promoter was induced >40-fold, while expression from the proximal promoter was not induced at all, paralleling the effects of exercise. Absolute expression from the alternative promoter is at baseline much less than expression from the proximal promoter; after clenbuterol treatment, however, alternative expression surpasses that of the proximal promoter (Fig. S3). The induction of PGC-1α from the alternative promoter was seen as early as 4 h, peaked at 6–8 h, and abated by 16 h after injection (Fig. 2G). Western blot analysis of muscle extracts from PBS- and clenbuterol-injected mice demonstrated strong induction of PGC-1α (Fig. 2F). Hence, as with exercise, β-adrenergic signaling induces expression of PGC-1α specifically from the alternative promoter.

PGC-1α acts in an autoregulatory loop to stimulate its own transcription (37). To test whether this autoregulatory loop was activated by β-adrenergic stimulation, PGC-1α MKO mice were injected with clenbuterol (1 mg/kg), and expression of the truncated (nonfunctional) PGC-1α transcripts were measured by qPCR with primers specific for either the alternative promoter 5′ UTR or the common 3′ UTR. PGC-1α protein was absent in muscles from MKO mice, even after clenbuterol treatment (Fig. 2F). Despite this, PGC-1α mRNA was robustly induced by clenbuterol in MKO mice (Fig. 2H). Hence β-adrenergic signaling on the PGC-1α promoter is independent of PGC-1α itself.

To investigate how β-adrenergic stimulation induces PGC-1α expression, a 4-kb region spanning the alternative PGC-1α

Fig. 3. β-adrenergic signaling activates the alternative PGC-1α promoter, via activation of a conserved cAMP responsive element. (A) Luciferase activity in muscles electroporated with alternative (Left) or proximal (Right) PGC-1α promoter-luciferase constructs, 6 h after i.p. injection with clenbuterol (1 mg/kg) or saline control. n = 6 muscles per group. (B) Luciferase activity in muscles electroporated with the indicated deletion of the alternative PGC-1α promoter-luciferase construct, 6 h after injection with clenbuterol or control. n = 6 muscles per group. Homology among mammals, and CRE sequences (in red), are shown at Bottom. Data are presented as mean ± SEM. *, P < 0.05 vs. control. †, P < 0.05 vs. intact promoter clenbuterol treated.

promoter was cloned upstream of a luciferase reporter gene. This construct was then transfected by electroporation directly into the tibialis anterior muscle of wild type mice. After 4 days, clenbuterol (1 mg/kg) vs. saline control was injected i.p. into the mice, and 6 h later the tibialis anterior muscles were harvested and luciferase activity was measured. As shown in Fig. 3A Left, clenbuterol treatment led to an 8-fold induction of luciferase activity, compared to saline control. Hence, β-adrenergic stimulation activates the PGC-1α alternative promoter in vivo. In contrast, β-adrenergic activity had no effect on the proximal PGC-1α promoter (Fig. 3A Right).

Comparison of the proximal and alternative PGC-1α promoters by sequence alignment did not reveal significant similarities, suggesting that the two promoters did not arise by gene duplication. Both promoters, however, contain a number of common consensus binding sites for various skeletal muscle-specific transcription factors like MEF2, MyoD, and myogenin, likely explaining the expression of both promoters in skeletal muscle (Fig. S4). To test which sequences in the PGC-1α alternative promoter render it susceptible to β-adrenergic stimulation, serial deletions were generated in the promoter construct. Electroporation of these plasmids into the tibialis anterior muscle, followed by measurement of luciferase activity, revealed that even a small deletion upstream of the transcriptional start site abrogated sensitivity to β-adrenergic signaling (Fig. 3B). The canonical signaling pathway for β-adrenergic stimulation acts through G protein Receptor signaling, adenylase cyclase, cAMP, protein kinase A, and ultimately through activation of cAMP responsive elements (CREs) in promoters of target genes. The short region of the PGC-1α alternative promoter identified above contains a consensus CRE, which is conserved across mammals (Fig. 3B). Mutation of this site almost abrogated the ability of clenbuterol to induce the PGC-1α alternative transcript (Fig. 3B). Hence, β-adrenergic signaling induces expression of PGC-1α via activation of a conserved CRE in the alternative promoter, most likely via canonical cAMP signaling.

β-Adrenergic Stimulation Induces VEGF in Skeletal Muscle via PGC-1α.

To test whether β-adrenergic signaling regulates an angiogenic program in skeletal muscle, mice were injected intraperitoneally with clenbuterol (1 mg/kg), and 6 h later RNA was prepared from
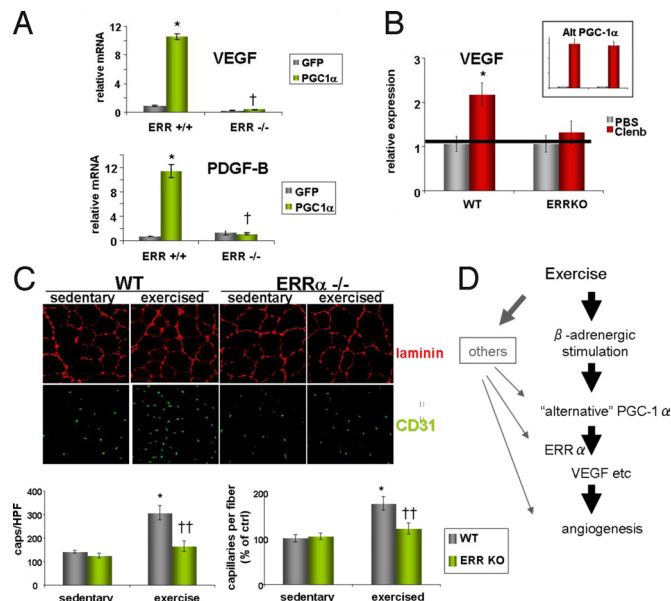
Fig. 4. β-adrenergic stimulation induces an angiogenic program in skeletal muscle via PGC-1α. (*A*) mRNA expression of the indicated angiogenic factors in quadriceps of wild-type mice 6 h after injection of clenbuterol (1 mg/kg). (*B*) mRNA expression of the same factors as in *A* in quadriceps of wild-type (WT) and MCK-PGC-1α(TG) mice. (*C*) Total PGC-1α (*Left*) and VEGF (*Right*) mRNA expression in quadriceps of mice after 16 h of voluntary running, injected with either propranolol or PBS (as indicated) 30 min before initiating the running. (*D*) Relative mRNA expression of VEGF (*Left*) and PDK4 (*Right*), in quadriceps of wild-type (WT) and PGC-1α −/− mice, 6 h after PBS (gray) or clenbuterol (green) injection. (*E*) As in *D*, with PGC-1α MKO mice. (*F*) Affymetrix microarray analysis of 2 WT and 2 −/− animals in *D*. *Left*, all genes with present calls and induced >2-fold by clenbuterol injection are shown. Red and blue indicate elevated and reduced expression, respectively. *Right*, representative genes. *n* = 3 per group for all except *E*. Data are presented as mean ± SEM. ∗, P < 0.05 vs. control. †, P < 0.05 vs. WT clenbuterol treated. ††, P < 0.05 vs. 16 h run.

Fig. 5. Exercise-induced angiogenesis requires ERRα. (*A*) Relative mRNA expression of VEGF (*Top*) and PDGF-B (*Bottom*), in differentiated primary skeletal myocytes isolated from wild-type (ERR +/+) or ERR −/− mice, 48 h after infection with adenovirus expressing PGC-1α or GFP control. *n* = 3 per group. (*B*) Relative mRNA expression of VEGF (and alternative PGC-1α: *Inset*) in quadriceps of wild-type (WT) and ERRα −/− mice, 6 h after PBS (gray) or clenbuterol (red) injection. (*C*) Capillary density, as determined in Fig. 1, from wild-type and ERRα −/− mice, either after 14 days of voluntary running, or sedentary controls. *Top*, representative immunostains. *Bottom*, quantification of capillaries/HPF (*Left*) and capillaries/fiber (*Right*). *n* = 5 per group. (*D*) Proposed model for part of the mechanism underpinning exercise-induced angiogenesis. See text for details. All data are presented as mean ± SEM. ∗, P < 0.05 vs. control. †, P < 0.05 vs. WT cells + PGC-1α. ††, P < 0.05 vs. WT exercised mice.

quadriceps muscles and the expression of various known angiogenic factors was measured by qPCR. As shown in Fig. 4*A*, the expression of a number of angiogenic factors, including VEGF, PDGF-B, and angiopoietin 2, was strongly induced by clenbuterol, while the expression of other angiogenic factors such as basic FGF and PDGF-A was repressed or unchanged. β-adrenergic stimulation thus triggers a broad reprogramming of angiogenic factor expression in skeletal muscle, including most notably the induction of VEGF. This reprogramming is strikingly similar to that induced by transgenic expression of PGC-1α in skeletal muscle (Fig. 4*B*), strongly suggesting a common mechanism.

To test this notion, mice were treated with propranolol (10 mg/kg), a β-adrenergic receptor blocker, and then allowed to run on in-cage voluntary wheels. After 16 h of running, levels of PGC-1α and VEGF expression were measured in quadriceps muscle. As shown in Fig. 4*C*, the expression of both PGC-1α and VEGF was strongly induced after 16 h of exercise in mice that were injected with saline control (green bars); in contrast, the induction of both VEGF and PGC-1α was significantly blunted in the presence of propranolol (red bars). Importantly, propranolol had no effect on the ability or willingness of mice to run on voluntary wheels. Hence, β-adrenergic stimulation mediates a large part of the induction of PGC-1α and VEGF by exercise.

Next, to investigate if PGC-1α mediates adrenergic induction of VEGF, PGC-1α −/− mice were injected with clenbuterol, and 6 h later quadriceps were isolated. Whereas clenbuterol induced VEGF expression 2.5-fold in wild-type animals, the induction of VEGF was abrogated in PGC-1α −/− mice (Fig. 4*D Left*). The same was true in PGC-1α MKO mice, indicating that the defect in signaling is intrinsic to the myocyte compartment (Fig. 4*E Left*). Consistent with these findings, Leick et al. recently demonstrated that the mild induction of VEGF seen after chronic forced exercise

was not observed in whole-body PGC-1α −/− mice (38). Together, these data demonstrate that exercise and β-adrenergic signaling induces VEGF expression in skeletal myocytes via PGC-1α.

Interestingly, the induction of some genes by clenbuterol is not affected in PGC-1α −/− mice. For example, pyruvate dehydrogenase kinase (PDK)-4 is still induced by clenbuterol in both PGC-1α −/− and MKO animals (Fig. 4 *D* and *E*, *Right*). Microarray analyses (Fig. 5*F*) revealed that the induction of approximately one-third of all genes induced by clenbuterol (generally the ones that were more strongly induced) was blocked in PGC-1α −/− animals, while the induction of the rest was unaffected. PGC-1α-dependent genes were strongly enriched for proteins involved in fatty acid oxidation (Fig. 4*F*), compared to PGC-1α-independent genes. Hence, PGC-1α mediates an important subset of the genomic effects of β-adrenergic stimulation in skeletal muscle.

ERRα and Exercise-Induced Angiogenesis. We showed previously that PGC-1α regulates VEGF by coactivating the orphan nuclear receptor ERRα on a number of conserved sites in an enhancer in the first intron of the VEGF gene (26). To test if the induction of VEGF by PGC-1α in skeletal muscle cells was entirely dependent on this ERRα pathway, primary skeletal muscle cells were isolated from ERRα −/− animals and wild-type controls. The cells were made to differentiate into myotubes in cell culture, and were infected with adenovirus encoding for PGC-1α, or GFP control. Infection of wildtype myotubes with PGC-1α virus induced expression of VEGF >10-fold, compared to control virus (Fig. 5*A Top*, left bars). In contrast, PGC-1α failed to have any impact on VEGF expression in ERRα −/− myotubes (Fig. 5*A Top*, right bars). The same was true of the induction by PGC-1α of PDGF-B (Fig. 5*A Bottom*).

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Thus, in skeletal muscle cells, PGC-1α induces VEGF and PDGF-B via ERRα.

To test the role of ERRα in β-adrenergic-mediated induction of VEGF in vivo, ERRα −/− mice (kindly provided by Shamina Rangwala, Novartis) and wild-type controls were injected with either PBS or clenbuterol. After 6 h, VEGF expression was measured in quadriceps by qPCR. As shown in Fig. 5B, the induction of VEGF by β-adrenergic signaling was abrogated in ERRα −/− mice. Hence, β-adrenergic signaling acts through PGC-1α to induce VEGF.

These data, combined with those provided in the previous section, suggest that alternative forms of PGC-1α are responsible for coactivating ERRα to induce VEGF expression. To test the ability of alternative PGC-1α to coactivate ERRα, PGC-1α1 and PGC-1α2 (the predominant alternative form) were expressed by transient transfection in 10T1/2 fibroblasts, and expression was confirmed by Western blot analysis (Fig. S5C). Cotransfection with ERRα and a luciferase-reporter construct containing concatemerized ERRα-binding sites revealed >40-fold induction by PGC-1α2 (Fig. S5A). Similarly, cotransfection with a GAL4-ERRα fusion protein and a luciferase-reporter construct containing concatemerized GAL4-binding sites also showed >40-fold induction by PGC-1α2 (Fig. S5B). Finally, cotransfection with a reporter construct containing the VEGF enhancer that we described (26) showed 5-fold induction by PGC-1α2, and mutation of key ERRα-binding sites in the enhancer abrogated the induction (Fig. S6). Hence PGC-1α2 coactivates ERRα to induce the VEGF enhancer.

Lastly, if exercise induces angiogenesis in skeletal muscle via PGC-1α-mediated induction of VEGF, and if the latter requires ERRα, then exercise-induced angiogenesis should be dependent on ERRα. To test this, ERRα −/− mice and wild-type controls were placed in cages containing voluntary running wheels. After 14 days, quadriceps were removed, and capillary density was quantified by immuno-histochemistry, as above. As shown in Fig. 5B, voluntary running markedly induced angiogenesis in wild-type mice. In sharp contrast, almost no changes in capillary density were seen in ERRα −/− mice. Hence, endurance exercise-induced angiogenesis requires ERRα.

Discussion

Together, the data presented here demonstrate the existence of a pathway that contributes to exercise-induced angiogenesis (Fig. 5C): exercise activates β-adrenergic signaling, leading in skeletal muscle to robust induction of PGC-1α from an alternative promoter; PGC-1α then acts through the orphan nuclear receptor ERRα to activate a broad program of angiogenesis, including the induction of VEGF; these angiogenic factors in turn orchestrate the highly complex process of physiological blood vessel formation.

The prevailing paradigm to explain exercise-induced angiogenesis has been that increased metabolic demands in exercising muscle cause a supply/demand mismatch. This mismatch causes hypoxia and/or ATP deficiency, leading to activation of HIF-1 and/or AMPK, respectively (7, 15–17). However, recent data do not entirely support this notion. Mice lacking HIF-1α in skeletal muscle have elevated, rather than lower microvascular densities, even at rest (18), and mice transgenically expressing a dominant-negative AMPK in skeletal muscle have intact exercise-induced angiogenesis (19). The data shown here strongly suggest a different (although not mutually exclusive) paradigm, whereby upstream β-adrenergic signaling, likely stemming from increased nerve activity, triggers angiogenesis. In this scenario, angiogenesis is triggered by the anticipated need for higher metabolic efficiency heralded by repetitive nerve stimulation. It is likely that this pathway also interacts with metabolic perturbations wrought by exercise. It will now be of interest to better understand how upstream signals are controlled locally, and how metabolic status integrates into the PGC-1 pathway.

The role of β-adrenergic signaling in angiogenesis has not been studied extensively. Iaccarino et al. recently showed that activation of β2-adrenergic receptors was proangiogenic in skeletal muscle (39). Thaker et al. also recently indicated that β-adrenergic stimulation, triggered by stress, increases tumor angiogenesis and worsens tumor burden in a mouse model of ovarian carcinoma (40, 41). Interestingly, an important role for β-adrenergic signaling in exercise-induced angiogenesis suggests that the use of β-blockers in patients with PAD might block some of the benefits of exercise. β-adrenergic blockers are widely used to treat patients with coronary artery disease (CAD), and patients with PAD usually have concurrent CAD. Anecdotal evidence and small trials have suggested that the use of β-blocking agents can worsen symptoms and function in patients with PAD (42). Recent trials have suggested that the use of β-blockers is safe, but these trials were small and, more importantly, of short duration (43). The long-term effects of β-blocking agents in patients with PAD thus remain uncertain (43).

The data presented demonstrate a key role for PGC-1α in exercise-induced angiogenesis, a normal physiological process. Angiogenesis is highly complex, involving multiple cells types and signals that must be coordinated in both space and time. The mechanisms by which angiogenesis occurs have been studied extensively in pathologic contexts, ranging from tumor neovascularization to proliferative retinopathy. Physiologic angiogenesis, however, has been studied much less extensively. It is likely that these two processes are not identical. PGC-1α orchestrates complex biologic programs in various tissues, ranging from mitochondrial biogenesis to gluconeogenesis (25). The data here suggest the possibility that PGC-1α may also be an orchestrator of physiological angiogenesis in general. It will be of great interest, therefore, to test the role of PGC-1α in other angiogenic processes that occur in adult tissues, such as endometrial angiogenesis during the oestrus cycle. It will also be of interest to investigate the precise cascade of events triggered by PGC-1α that leads to the formation of new and functional blood vessels, and how that sequence of events differs from that initiated in pathologic contexts, such as HIF-1 activation in tumor cells.

Why the induction of PGC-1α should occur via the striking activation of an alternative promoter is not clear. It is possible that the marginal changes in the N terminus of these proteins confer specificity on the angiogenic program. PGC-1α originating at the proximal promoter, however, is clearly able to strongly induce VEGF and other angiogenic factors, indicating that induction of these factors is not the unique purview of alternative forms of PGC-1α. Alternatively, the use of the alternative promoter may simply allow tissue-specific regulation, since the alternative promoter is so specific to skeletal muscle. Interestingly, BAT is the only other tissue in which the alternative PGC-1α promoter appears to be active, and BAT also undergoes angiogenesis in response to β-adrenergic stimulation, in a HIF-independent fashion (44). This suggests that PGC-1α transcribed from the alternative promoter may mediate angiogenesis in BAT as well.

It is important to note that pathways for exercise-induced angiogenesis other than that outlined in Fig. 5C likely also exist. A large number of signaling pathways are activated during exercise, including the calcineurin phosphatase and calmodulin-modulated kinase, the AMP-sensitive AMP Kinase (AMPK), the stress-responsive p38 MAPK, and the production of reactive oxygen species (48–50). All of these pathways are known to impinge on PGC-1α (51–54), and may therefore contribute to the activation of PGC-1α in exercise. Moreover, PGC-1α-independent pathways may also exist. Nevertheless, the pathway described here appears to contribute significantly to exercise-induced angiogenesis.

Exercise is a staple in the treatment of PAD. Diabetes is a strong risk factor for PAD, and PGC-1α is repressed in diabetic skeletal muscle in both rodents and humans (45–47), suggesting that

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PGC-1α insufficiency may predispose to, or worsen PAD. The data presented here, combined with our previous studies (26), suggest that the benefits of exercise in PAD may also in part be mediated by PGC-1α. Tapping into this preexisting program may therefore provide a therapeutic potential for treating ischemic disease in its many guises. In summary, we describe a pathway to explain exercise-induced angiogenesis. The pathway provides insights into mechanisms of physiological angiogenesis, and may provide an important target for therapeutic modalities aimed at increasing vascular density.

Materials and Methods

All animal experiments were performed according to procedures approved by the Institutional Animal Care and Use Committee. All reagents were from Sigma, unless otherwise indicated. All results are expressed as means ± SEM. Two-tailed independent Student's *t* tests were used to determine all *P* values. See *SI Text* for further details.

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